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Activation by Autophosphorylation or cGMP Binding Produces a Similar Apparent Conformational Change in cGMP-dependent Protein Kinase*

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Binding of cyclic nucleotide to or autophosphorylation of cGMP-dependent protein kinase (PKG) activates this kinase, but the molecular mechanism of activation for either process is unknown. Activation of PKG by cGMP binding produces a conformational change in the enzyme (Chu, D.-M., Corbin, J. D., Grimes, K. A., and Francis, S. H. (1997) *J. Biol. Chem.* 272, 31922–31928; Zhao, J., Trewhella, J., Corbin, J., Francis, S., Mitchell, R., Brushia, R., and Walsh, D. (1997) *J. Biol. Chem.* 272, 39129–31936). In the present studies, activation of type Iβ PKG by either autophosphorylation or cGMP-binding alone causes (i) an electronegative charge shift on ion exchange chromatography, (ii) a similar increase (~3.5 Å) in the Stokes radius as determined by gel filtration chromatography, and (iii) a similar decrease in the mobility of the enzyme on native gel electrophoresis. Consistent with these results, cGMP binding increases the rate of phosphoprotein phosphatase-1 catalyzed dephosphorylation of PKG which is autophosphorylated only at Ser-63 (not activated); however, dephosphorylation of PKG that is highly autophosphorylated (activated) is not stimulated by cGMP. The combined results suggest that activation of PKG by either autophosphorylation or cGMP binding alone produces a similar apparent elongation of the enzyme, implying that either process activates the enzyme by a similar molecular mechanism.

Protein phosphorylation plays key roles in regulating protein function in myriad biological processes. Activation of the protein kinases that catalyze these protein phosphorylations is certainly one of the major mechanisms by which cellular functions are controlled. A particular protein kinase not only phosphorylates one or more cellular proteins (heterophosphorylation),[1] but it commonly phosphorylates itself as well, a process termed autophosphorylation. Protein kinase autophosphorylation is functionally important, since it frequently alters kinase function, *e.g.* by increasing the catalytic activity, increasing the affinity for allosteric ligand binding, or increasing the kinase binding to cellular proteins such as those containing SH2 domains. Many protein kinases are activated by either allosteric ligand binding or autophosphorylation (1). In some cases, ligand binding stimulates the rates of both autophosphorylation and heterophosphorylation. Furthermore, autophosphorylation of some protein kinases increases both the binding affinity for regulatory ligand(s) and the kinase catalytic activity. Therefore, in these instances ligand binding and autophosphorylation act in concert to produce an enhanced activation. The mechanisms of activation of protein kinases by these two processes are still unknown. Although these processes seem quite different, it seems reasonable that similar molecular perturbations may be involved to produce the final activation state for each process.

Recently, activation of cGMP-dependent protein kinase (PKG)[2] by cGMP binding was shown to cause a conformational change in the enzyme (2, 3). The results show that an increased net negative surface charge and elongation of the enzyme occurs when PKG binds cGMP. These effects are apparently associated with a conformational change that relieves the interaction of the autoinhibitory domain with the catalytic site, thereby activating the protein kinase. Like many other protein kinases, PKG and type II cAMP-dependent protein kinase (PKA) undergo autophosphorylation, and this process affects the kinetic properties of each enzyme (4–18). The type Iα or type Iβ PKGs are autophosphorylated at sites in or near their autoinhibitory domains, and this modification of the PKGs increases the kinase activity (minus cyclic nucleotide) (15, 18). However, the mechanism whereby autophosphorylation activates the cyclic nucleotide-dependent protein kinases, as well as other protein kinases, is not known (19, 20). Since the autophosphorylation sites in type I PKGs are located in the autoinhibitory domain (12, 17, 18), it is thought that autophosphorylation, like cGMP binding, may induce a conformational change that disrupts the autoinhibition, thus activating these protein kinases. Whether or not autophosphorylation and allosteric ligand binding could activate the protein kinases by producing a similar conformational change has not been studied. Autophosphorylation of the intracellular tyrosine kinase domain of the epidermal growth factor receptor causes a 3–5-Å increase in the apparent Stokes radius of this enzyme (21). Studies of the crystal structure of glycogen phosphorylase reveals that activation by phosphorylation or by the ligand activator, adenosine 5′-monophosphate, causes the same overall

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[1] The term heterophosphorylation is derived in part from the Greek *heteros* (other, different), which is compared with the Greek *autos* (self). Thus, heterophosphorylation refers to phosphorylation of proteins, peptides, or substances other than the kinase itself or a subunit(s) of the kinase itself. The terms heterophosphorylation and autophosphorylation are related in the same way as are heterophagy and autophagy.

[2] The abbreviations used are: PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; RIIα, regulatory subunit of type IIα PKA; 8-Br-PET-cGMP, β-phenyl-1,N^2 etheno-8-bromo-cGMP.

conformational change in this protein (22). Although the phosphate is not introduced by autophosphorylation in the case of phosphorylase, the same guiding principle will be applied for the present studies, *i.e.* that activation of PKG by a phosphorylation event, *i.e.* autophosphorylation, or cyclic nucleotide binding produces a similar conformational change in this enzyme.

To assess possible conformational changes of type Iβ PKG produced by cGMP binding and/or autophosphorylation, four different techniques have been used: (i) determining the PKG elution position on ion exchange columns to detect potential difference in surface charge of the enzyme, (ii) determining the elution position of the PKG on gel filtration columns to detect potential difference in mass and shape of the enzyme, (iii) determining the PKG mobility on native gel electrophoresis to detect potential differences in surface charge and shape of the enzyme, and (iv) determining the sensitivity of autophosphorylated PKG to phosphoprotein phosphatase-1 action. The present studies provide the first evidence for a similar molecular activation of a protein kinase by ligand binding and autophosphorylation.

EXPERIMENTAL PROCEDURES

Purification of PKG and Protein Kinase Assay—Bovine aorta type Iβ PKG was purified to homogeneity as described by Francis *et al.* (23). The heterophosphorylation kinase activity of PKG was determined by the phosphocellulose paper assay using heptapeptide substrate (RKRSRAE) as described previously (16).

Preparation and [^{3}H]cAMP Binding Assay of Cyclic Nucleotide-free Regulatory Subunit of Type IIα PKA—The dimeric regulatory subunit of bovine heart type IIα PKA (RIIα) was purified according to the method of Corbin *et al.* (24). The binding activity of RIIα was measured by [^{3}H]cAMP binding assay as described previously (25). Urea denaturation of RIIα to remove cyclic nucleotides was performed as described by Poteet-Smith *et al.* (26). Like native RIIα, this urea-treated cAMP-free RIIα exhibited two active cAMP-binding sites, inhibited the catalytic subunit of PKA stoichiometrically, and had a dimeric structure as verified by determination of the sedimentation coefficient.

Measurement of cAMP and cGMP—The cyclic nucleotide contents of the purified proteins were determined using the modified version of the cyclic nucleotide assay previously described by Corbin *et al.* (27) and Chu *et al.* (2).

Preparation and Purification of Phosphorylated PKG and RIIα—Autophosphorylated PKG was prepared by incubating purified type Iβ PKG (38 μg/ml) with 4.8 mM magnesium acetate, 100 μM [γ-^{32}P]ATP, and 50 μM cAMP at 30 °C for various times. To assess ^{32}P incorporation, an aliquot (10 μl) of this reaction mixture was spotted onto phosphocellulose paper, washed with four changes of 75 mM phosphoric acid, dried, and counted. After incubation, 10 mM EDTA was added to stop the reaction. To remove [γ-^{32}P]ATP and cAMP, the sample was chromatographed at 4 °C either on a Sephadex G-25 (superfine) column (0.9 × 11 cm) equilibrated in 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, and 0.1 M NaCl or on a Sephacryl S-200 column (0.9 × 56 cm) equilibrated in 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 25 mM β-mercaptoethanol (KPEM), and 40 mM NaCl. Fractions of 0.5 ml were collected. Aliquots (10 μl) of each fraction were counted in 1 ml of aqueous scintillant to determine the radiolabeled phosphate content, and the protein content of each fraction was measured by absorbance at 280 nm. Fractions containing the peak [^{32}P]PKG were pooled for the experiments. To prepare autophosphorylated type II regulatory subunit of PKA (RIIα), the protein (10 μM) was incubated with 3.5 mM magnesium acetate, 35 μM [γ-^{32}P]ATP, and 10 μM cAMP at 30 °C for 20 min. The same procedures were performed as described above to remove reaction components and to determine ^{32}P incorporation and purification of autophosphorylated RIIα. Purified RIIα contains a trace contamination of catalytic subunit of PKA, which is sufficient to obtain partially autophosphorylated RIIα at Ser-95 (6–8). Under the conditions used, the phosphate incorporation was ~0.75 mol of ^{32}P/mol of RIIα subunit.

Ion Exchange Chromatography—A mixture of purified unphosphorylated and ^{32}P-labeled autophosphorylated PKG that had been preincubated in the absence or presence of cGMP was applied to a DEAE-Sephacel column (0.9 × 10 cm) equilibrated in KPEM at 4 °C and analyzed as described earlier (2). Aliquots of each fraction were counted to determine the phosphate content of the kinase. The unphosphorylated PKG was used as an internal control to normalize the elution position of ^{32}P-autophosphorylated PKG or cGMP-bound PKG. For experiments utilizing cGMP-bound PKG, the column was pre-equilibrated with KPEM containing 100 μM cGMP, and the KPEM elution buffer also contained 100 μM cGMP to assure that the cGMP-binding sites of the enzyme remained saturated with cGMP during chromatography. The cGMP concentration in the KPEM buffer was determined by measuring absorbance (molar extinction coefficient for cGMP, ϵ_{252} = 13,700).

Gel Filtration Chromatography—Purified unphosphorylated or ^{32}P-labeled autophosphorylated PKG that had been preincubated in the absence or presence of 100 μM cGMP was combined with crystalline catalase (4 mg) in a total volume of 500 μl. The mixture was shaken gently to dissolve the catalase and then loaded onto a Sephacryl S-300 column (0.9 × 168 cm) equilibrated with KPEM buffer containing 0.1 M NaCl at 4 °C. The catalase served as an internal standard for all of the gel chromatographies. Fractions were collected and analyzed as described earlier (2). To determine the phosphate content of the enzyme, the radiolabeled phosphate was measured in aliquots of each fraction by scintillation counting. In experiments in which PKG was presaturated with cGMP, the column was pre-equilibrated with 100 μM cGMP, and the column running buffer also contained 100 μM cGMP. Because cGMP in the column buffer interfered with the absorbance at 280 nm, absorbance at 400 nm was used to determine the elution position of catalase for those experiments involving cGMP-bound PKG. To compare the effect of different wavelength absorbances on the apparent elution position of catalase, absorbances at both 280 and 400 nm were compared for several purified PKG experiments, and the elution position of the catalase peak was the same by either technique.

Determination of Stokes Radius, Sedimentation Coefficient, and Molecular Weight—Apparent Stokes radii of purified unphosphorylated and autophosphorylated PKG in the presence and absence of cGMP were determined by the method described previously (2, 16). The sedimentation coefficients of the enzymes were determined as described earlier (2). The apparent molecular weights, frictional ratios, and axial ratios were calculated according to the method of Siegel and Monty (28), together with the procedures of Cohn and Edsall (29).

Dephosphorylation of Type Iβ PKG by Phosphoprotein Phosphatase-1—Autophosphorylated PKG was produced by incubating 130 μl of 0.1 mg/ml type Iβ PKG in 10 mM potassium phosphate, 2 mM EDTA, and 25 mM β-mercaptoethanol in the presence of 40 μM cAMP, 5 mM magnesium acetate, and 100 μM [^{32}P]ATP at 30 °C for 7 min (partially autophosphorylated) or 2 h (highly autophosphorylated). Partially autophosphorylated enzyme contained nearly stoichiometric phosphate at Ser-63, whereas highly autophosphorylated enzyme contained approximately stoichiometric amounts of phosphate at Ser-63 and was nearly saturated at Ser-79. The autophosphorylated PKG was chromatographed on a Sephacryl S-200 column (0.9 × 48 cm) equilibrated in 40 mM Tris (pH 7.5), 25 mM β-mercaptoethanol, and 2 mg/ml bovine serum albumin, and the peak fractions of activity were pooled for use in the phosphoprotein phosphatase reaction. 0.025 unit/ml phosphoprotein phosphatase-1 catalytic subunit (Promega) was preincubated at 4 °C for 15 min in 400 μl of 20 mM Tris (pH 7.5), 2 mg/ml bovine serum albumin, in the absence or presence of 15 μM cGMP or 50 μM cAMP. 192 μl of a final concentration of 1 μM partially or highly autophosphorylated PKG were added, and the mixture was incubated at 30 °C for varying amounts of time. 15-μl aliquots of the incubation mixture were removed at certain time points and placed in a tube containing a final concentration of 1 mg/ml bovine serum albumin and 10% trichloroacetic acid in a total volume of 540 μl. The tubes were vortexed, incubated at 4 °C for 1 h, and centrifuged for 10 min at 10,000 × *g*. 450-μl aliquots of the supernatant were counted in the scintillation counter to determine the total amount of free phosphate present. 15-μl aliquots of the incubation mixture were counted at each time point to determine the total amount of phosphate present. The amount of free phosphate was divided by the total amount of phosphate to determine the percentage of phosphate released.

Native Gel Electrophoresis—The enzymes were electrophoresed on a 9.5% polyacrylamide gel and 4% stacking gel without sodium dodecyl sulfate at 4 °C using constant current (~10 mA) for 5 h as previously described (2). The proteins were detected by Coomassie Brilliant Blue staining.

Materials—All materials were obtained as described earlier (2).

RESULTS AND DISCUSSION

Cyclic Nucleotide-free and cGMP-bound PKG—The cAMP and cGMP contents of the purified type Iβ PKG were measured

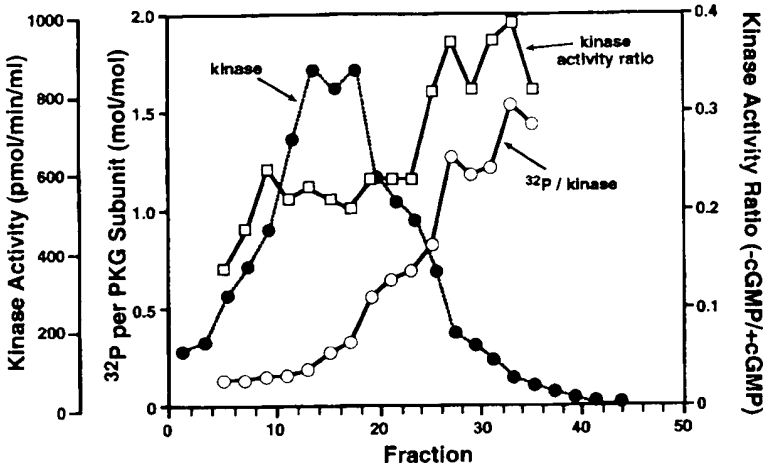

FIG. 1. **Effect of autophosphorylation of type Iβ PKG on its elution from DEAE-Sephacel chromatography.** A mixture of unphosphorylated enzyme (60 μg) and ^{32}P-labeled autophosphorylated enzyme (10 μg) in a volume of ~1 ml was loaded onto a DEAE-Sephacel column as described under "Experimental Procedures." Aliquots (20 μl) of fractions were assayed for kinase activity to determine the elution position of the enzymes, and aliquots (500 μl) of each fraction were counted in 5 ml aqueous scintillant to determine the phosphate content of the ^{32}P-labeled autophosphorylated enzyme. The protein kinase activity ratio for each fraction was expressed as the activity measured in the absence of cGMP divided by the activity obtained in the presence of 10 μM cGMP. The molarity of the kinase in the fraction was calculated from the specific enzyme activity (3 μmol/min/mg) of the purified enzyme used for the experiments. The results shown are representative of four separate experiments.

as described earlier (2). The cyclic nucleotide occupancy of cGMP-binding sites of purified PKG used for the following experiments was less than 3%. Previous studies suggest that the purified PKGs are not significantly phosphorylated at the autophosphorylation sites that alter catalytic activity or cyclic nucleotide-binding affinity (18). The purified PKG also had a very low basal kinase activity ratio (−/+ cGMP ~0.05). Therefore, the purified type Iβ PKG was considered to be cyclic nucleotide-free and not autophosphorylated. To prepare cGMP-bound PKG, the enzyme was incubated with unlabeled cGMP (~500-fold excess of cGMP-binding sites) at 4 °C overnight, and equal occupation of the two intrasubunit cGMP-binding sites was verified as described earlier (2). For native gel electrophoresis experiments, purified PKG was incubated with unlabeled cGMP or cGMP analog, 8-Br-PET-cGMP, as described above to obtain cyclic nucleotide-bound PKG.

Autophosphorylation of PKG—Autophosphorylated type Iβ PKG was prepared by incubating purified enzyme in the presence of cAMP, magnesium acetate, and [γ-^{32}P]ATP followed by gel filtration on a Sephacryl S-200 column to remove cAMP and other reactants as described under "Experimental Procedures." After overnight incubation (highly autophosphorylated), the enzyme contained ~2 mol of ^{32}P/mol of subunit with equal distribution of the phosphate between Ser-63 and Ser-79. The stoichiometry of 2 mol of phosphate incorporated per mol of subunit indicated that these sites were largely in the dephosphorylated state in the purified enzyme before autophosphorylation. The increased phosphate content of the enzyme increased the basal heterophosphorylation activity in the absence of cGMP by ~5-fold. When the PKG was incubated for only 7 min (partially autophosphorylated), the phosphate incorporation was ~0.5 mol of ^{32}P/mol of subunit, and basal heterophosphorylation activity did not increase. The results confirmed that autophosphorylation of type Iβ PKG at Ser-79, which is slowly autophosphorylated, causes the increase in kinase activity, whereas the rapid autophosphorylation at Ser-63 does not (18). Since autophosphorylation of PKG was performed in the presence of cAMP, it was necessary to verify that cyclic nucleotide was removed by the gel filtration step. The measured cAMP and cGMP contents of autophosphorylated enzyme were calculated to be less than 10% occupancy of cGMP-binding sites of the protein so that the autophosphorylated PKG Iβ used for subsequent experiments was essentially cyclic nucleotide-free unless cGMP was purposely included.

DEAE-Sephacel Anion Exchange Chromatography of Unphosphorylated, Autophosphorylated, and cGMP-bound PKG—When a mixture of predominantly purified unphosphorylated PKG and a trace amount of purified ^{32}P-radiolabeled autophosphorylated type Iβ PKG was chromatographed on a DEAE-Sephacel column as described under "Experimental Procedures," the peak of autophosphorylated enzyme activity eluted at higher ionic strength and was partially separated from the bulk of the unphosphorylated enzyme activity (Fig. 1). Fractions containing the ^{32}P-labeled enzyme showed a higher kinase activity ratio (−/+ cGMP ~0.4) than did fractions containing unphosphorylatec enzyme (−/+ cGMP ~ 0.1). The ^{32}P-labeled enzyme eluting at the highest ionic strength had the highest phosphate content (1.5 mol/subunit) and also had the highest kinase activity ratio (Fig. 1).

The autophosphorylated PKG used for the experiment of Fig. 1 was not quantitatively phosphorylated at the two autophosphorylation sites, Ser-63 and Ser-79; furthermore, separation of the unphosphorylated and phosphorylated PKG forms was not complete. To achieve optimum resolution of the unphosphorylated and phosphorylated PKGs, the respective enzymes were first chomatographed on DEAE-Sephacel as described in Fig. 1. In a subsequent experiment, PKG in fractions that eluted at the lowest ionic strength as shown in Fig. 1 were used for the unphosphorylated enzyme, and the PKG in enzyme fractions that eluted at the highest ionic strength were used for the phosphorylated PKG. As shown in Fig. 2*A*, a mixture of the unphosphorylated PKG and a trace amount of the ^{32}P-radiolabeled autophosphorylated type Iβ PKG was then rechromatographed on a DEAE-Sephacel column as described under "Experimental Procedures." The peak of autophosphorylated enzyme activity eluted at higher ionic strength and was well separated from the bulk of the unphosphorylated enzyme activity (Fig. 2, *top panel*). When the unphosphorylated PKG was preincubated with cGMP and chromatographed in the presence of buffer containing 100 μM cGMP, the peak of cGMP-bound enzyme activity also eluted at higher ionic strength (Fig. 2, compare kinase profiles in the *top* and *bottom panels*). However, there was no additive effect resulting from a combination of cGMP and autophosphorylation (Fig. 2, compare ^{32}P profiles

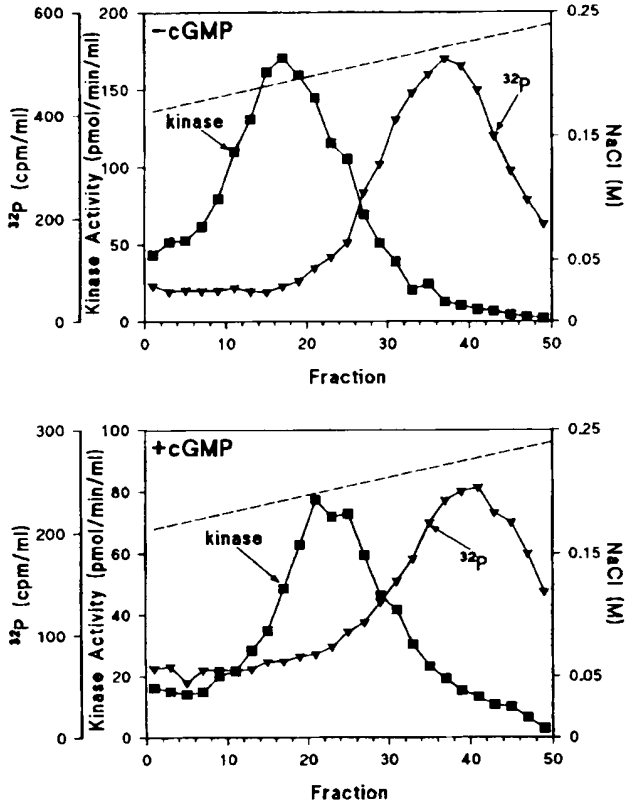

FIG. 2. **Effect of autophosphorylation and cGMP binding on DEAE-Sephacel chromatography of type Iβ PKG.** A mixture of the unphosphorylated PKG and ^{32}P-autophosphorylated PKG were chromatographed separately on DEAE-Sephacel columns as described in Fig. 1 and "Experimental Procedures." Then the unphosphorylated enzyme (fractions eluting at the lowest ionic strength, which would presumably contain the lowest level of phosphorylated enzyme) and phosphorylated enzyme (pooled fractions eluting at high ionic strength, which would presumably contain the highest level of phosphorylated enzyme) were mixed and rechromatographed on DEAE-Sephacel. *Top*, chromatographic profile of a mixture of unphosphorylated PKG (15 μg) and a trace amount of ^{32}P-labeled autophosphorylated PKG (0.4 μg) in the absence of cGMP. *Bottom*, chromatographic profile of a mixture of unphosphorylated enzyme (8 μg) and ^{32}P-labeled autophosphorylated enzyme (0.4 μg) that had been preincubated with cGMP (~500-fold excess of cGMP-binding sites). KPEM chromatography buffer contained 100 μM cGMP. Fractions were analyzed for kinase activity and ^{32}P content as described under "Experimental Procedures." The results are representative of four separate experiments.

in the *top* and *bottom panels*). The shift in elution position did not occur when PKG was partially autophosphorylated using a 7-min incubation (data not shown). Partial autophosphorylation of type Iβ PKG is associated with the incorporation of phosphate at Ser-63 (18) and not associated with an increase in basal kinase activity. However, when this partially autophosphorylated enzyme was preincubated with cGMP and chromatographed in the presence of buffer containing 100 μM cGMP, the peak of PKG activity was shifted to a higher ionic strength (data not shown) that was similar to the elution position of the unphosphorylated PKG in the presence of 100 μM cGMP.

The fact that autophosphorylated or cGMP-bound PKG eluted at higher ionic strength from the DEAE column than did the control enzyme indicated that either autophosphorylation or cGMP binding increased the net negative surface charge of the enzyme to produce an electronegative charge shift. Since the covalent phosphates or bound cGMP molecules possess negative charges, a surface location of either the phosphate introduced by autophosphorylation or the cyclic phosphate of the cGMP molecule could be responsible for this effect. However, several reports have suggested that negative charges of the bound cGMP molecules in PKG are not near the surface of the protein (30–32). Since the autophosphorylated enzyme eluted at noticeably higher ionic strength than did the cGMP-bound enzyme, it cannot be ruled out that some of the charge shift produced by autophosphorylation is due to surface phosphates *per se*. It seems plausible that activation of PKG by autophosphorylation and/or cGMP binding causes a similar conformational change that results in increased net surface electronegativity of each isoform, and that the greater DEAE column shift produced by autophosphorylation is due to extra surface charges contributed by the phosphates. The fact that the effects of cGMP and autophosphorylation were not additive is consistent with this proposal.

Sephacryl S-300 Gel Filtration Chromatography of Unphosphorylated, Autophosphorylated, and cGMP-bound PKG—Unphosphorylated and highly autophosphorylated type Iβ PKGs were chromatographed on a Sephacryl S-300 gel filtration column in the presence of the internal standard catalase as described under "Experimental Procedures." The highly autophosphorylated PKG eluted earlier from the column than did the unphosphorylated form of this enzyme (Fig. 3, *A* and *B*). When the enzyme was preincubated with cGMP and chromatographed in the presence of buffer containing 100 μM cGMP, the kinase again eluted earlier than did the control, and there was no significant difference in elution position of this enzyme compared with the respective autophosphorylated enzyme (Fig. 3, compare *B* and *C*). There was no additional shift in elution position of the autophosphorylated PKG when chromatographed in the presence of cGMP (data not shown). Again, partial autophosphorylation (7-min incubation) of PKG did not produce a detectable shift in elution position on the S-300 column (data not shown). The results using gel filtration indicated that activation of PKG by autophosphorylation and/or cGMP binding produces a similar shift in elution position of the enzyme. As was shown for DEAE anion-exchange chromatography (see above), the combination of autophosphorylation and cGMP binding does not produce an additive shift. These results are consistent with *in vitro* kinase activation studies which indicate that in the presence of saturating concentration of cyclic nucleotides, autophosphorylation of PKG does not increase the kinase catalytic activity (18).

Physical Parameters of the PKG—The Stokes radii of different forms of type Iβ PKG were calculated from the results of gel filtration as described earlier (2). As can be seen in Table I, the Stokes radii of autophosphorylated and cGMP-bound PKGs were ~3.5 Å larger than the Stokes radii of the unphosphorylated enzyme, and there was no significant difference in the sedimentation coefficients for these autophosphorylated and unphosphorylated enzymes. The calculated apparent molecular weights indicated that the shifts in elution position of the PKG were not large enough to be due to oligomerization of the dimeric enzyme. Furthermore, the added mass of either the four bound cGMP molecules (367 Da each) or four phosphate groups (80 Da each) to the dimeric PKG would be insufficient to produce a size shift of this magnitude. The axial ratios (Table I) suggested that the autophosphorylated and cGMP-bound forms are more elongated proteins when compared with the unphosphorylated or cyclic nucleotide-free enzyme, and this elongation is reflected in an increase in the apparent Stokes radius. The findings from both anion exchange and gel filtration chromatography indicated that activation of type Iβ PKG by autophosphorylation or cGMP binding produces a similar apparent conformational change with elongation of the enzymes and increased net negative surface charge.

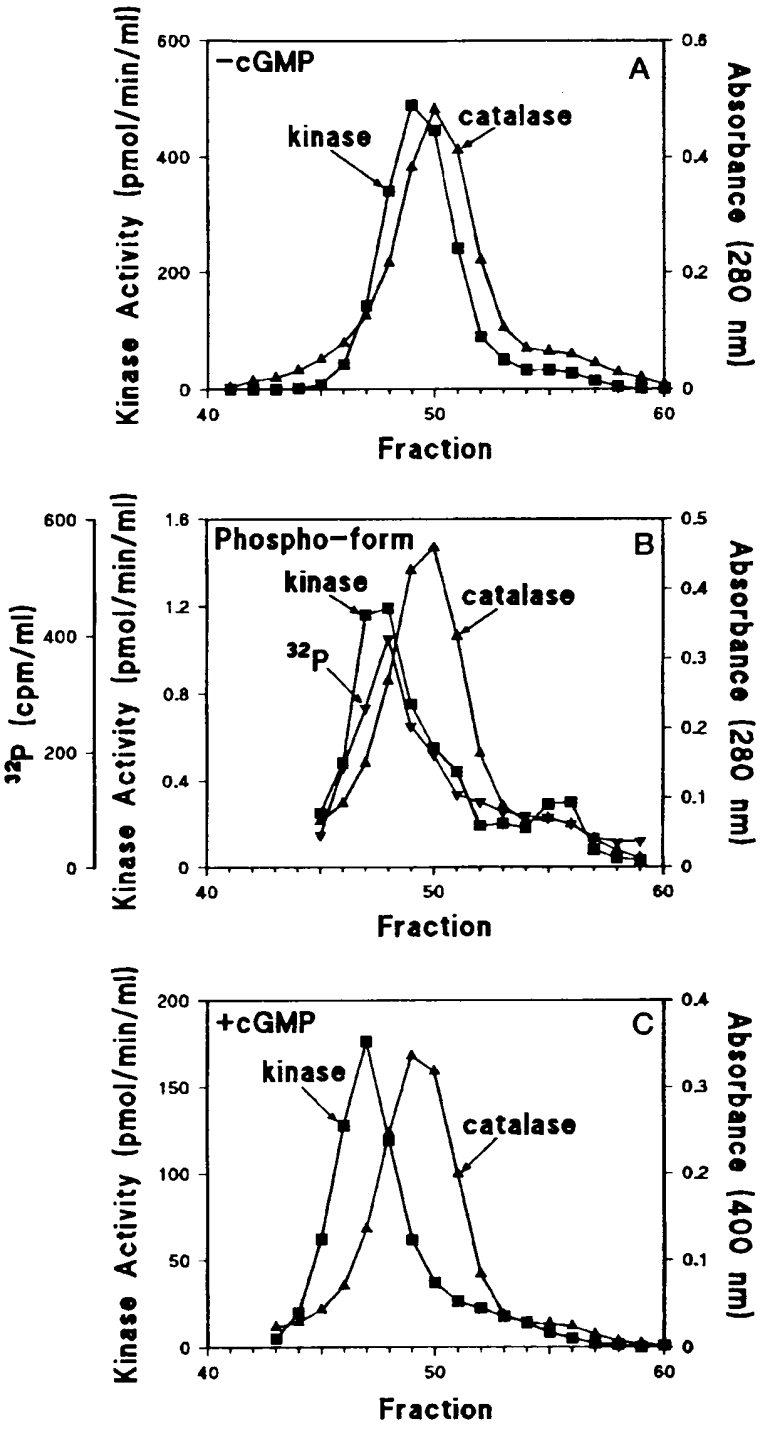

FIG. 3. **Effect of autophosphorylation and cGMP binding on Sephacryl S-300 gel filtration chromatography of type Iβ PKG.** *A*, a mixture of unphosphorylated enzyme (15 μg) and catalase (4 mg); *B*, a mixture of ^{32}P-labeled autophosphorylated enzyme (40 ng) and catalase (4 mg); or *C*, a mixture of unphosphorylated (cGMP-bound) enzyme (8 μg) and catalase (4 mg) was chromatographed in the presence of 100 μM cGMP and analyzed as described under "Experimental Procedures." The ^{32}P-labeled autophosphorylated PKG used in *panel B* was first chromatographed on Sephacryl S-300, and the enzyme fractions that eluted earliest were pooled and used for the rechromatography shown in *panel B*. Each panel is representative of four experiments and analyzed as described under "Experimental Procedures."

Native Gel Electrophoresis—Native gel electrophoresis was also used to detect differences in molecular parameters of unphosphorylated, autophosphorylated, and cyclic nucleotide-bound type Iβ PKG according to the method described by Chu *et al.* (2). Both highly autophosphorylated and 8-Br-PET-cGMP-bound PKGs exhibited the same mobility on native gels, and this mobility was less than that of the unphosphorylated form of the enzyme (Fig. 4*A*). PKG has a high affinity for 8-Br-PET-cGMP, and this analog has been shown to remain bound to the enzyme during native gel electrophoresis (2). When the enzymes were presaturated with cGMP (or 8-Br-PET-cGMP) and then electrophoresed in the presence of 100 μM cGMP, the mobility of the PKGs was the same irrespective of each treatment (Fig. 4*B*). This mobility was also the same as that obtained for either the autophosphorylated or cGMP analog-bound PKG in Fig. 4*A*. Both autophosphorylation and cGMP analog together did not produce an additive effect on mobility (Fig. 4*B*). The apparent increase in the surface electronegativity of autophosphorylated or cGMP-saturated PKG using DEAE-Sephacel chromatography would be predicted to increase the mobility of the enzyme toward the anode using native gel electrophoresis. However, the mobility of the type Iβ PKG in the gel is decreased and is consistent with a conformational change that increases the apparent size of the protein

TABLE I

Physical parameters of type Iβ unphosphorylated and autophosphorylated cGMP-dependent protein kinase in the absence and presence of cGMP

For Stokes radii or sedimentation coefficients, values are mean ± S.D. ($n > 3$).

Enzyme	Stokes radius	Sedimentation coefficient	Calculated M_r[a]	Frictional ratio	Axial ratio
	Å	$s_{20,w}$		f/f_o	*length/width*
cGMP-free	47.8 ± 0.2	7.1 ± 0.1	141,000	1.40	6.0
Phospho-form	50.7 ± 0.3[b]	6.8 ± 0.1	143,000	1.46	6.9
cGMP-bound	51.3 ± 0.1[b]	6.9 ± 0.1	147,000	1.47	7.0
Phospho-form + cGMP-bound	51.3 ± 0.2[b]	6.9 ± 0.1	147,000	1.47	7.0

[a] Molecular mass is calculated from Stokes radius and sedimentation coefficient (see text).
[b] Significantly greater than cGMP-free PKG Iβ value ($p < 0.005$).

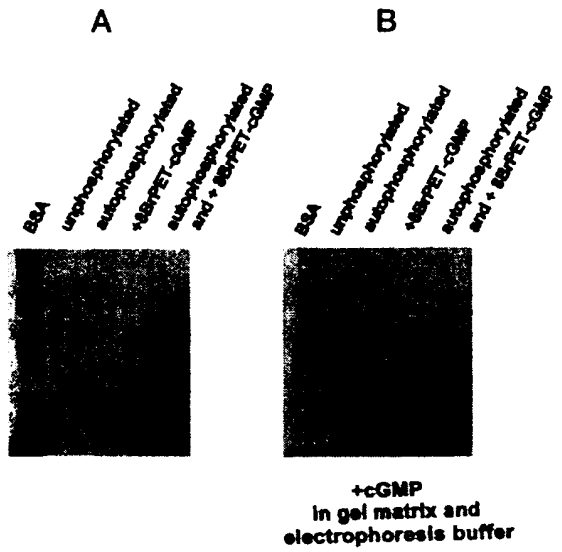

FIG. 4. **Effect of autophosphorylation and cyclic nucleotide binding on the mobility of type Iβ PKG on native gel electrophoresis.** *A*, unphosphorylated (cyclic nucleotide-free) (15 μg) and autophosphorylated (15 μg) enzymes were prepared as described under "Experimental Procedures." The enzymes were subjected to electrophoresis in the presence and absence of 8-Br-PET-cGMP on a 9.5% polyacrylamide gel in the absence of sodium dodecyl sulfate and stained with Coomassie Brilliant Blue as described under "Experimental Procedures." *B*, the same enzymes were analyzed on a 9.5% native polyacrylamide gel containing 100 μM cGMP. The results shown are representative of five separate experiments.

upon activation by either autophosphorylation or cyclic nucleotide binding. These results confirm the interpretation of the previous results from gel filtration analysis.

Effect of cAMP on the Regulatory Subunit of PKA—As described earlier (2), the cAMP-free RIIα of PKA was used as a control for each of the three approaches described above. The cAMP-free, autophosphorylated RIIα and the cAMP-bound RIIα were prepared as described under "Experimental Procedures" and then subjected to DEAE ion exchange chromatography, gel filtration chromatography, as well as native gel electrophoresis. Neither autophosphorylation nor cAMP binding produced a detectable electronegative charge shift on DEAE chromatography (data not shown). There was also no shift in elution position of autophosphorylated or cAMP-bound RIIα on gel filtration chromatography, and no mobility shift on native gel electrophoresis. The results indicated that introduction of extra charge or mass by addition of the phosphates or four cAMP molecules is not sufficient to change the surface charge or increase the mass to produce a shift of the RIIα using these procedures. A similar conformational change caused by autophosphorylation or cGMP binding is a more reasonable explanation for the findings using PKG.

Effect of Cyclic Nucleotide and Full Autophosphorylation on Dephosphorylation of PKG—Type Iβ PKG contains two autophosphorylation sites (Ser-63 and Ser-79) (17, 18). Autophosphorylation of Ser-63 occurs first, but significant enzyme activation occurs only after subsequent autophosphorylation of Ser-79 (18). Dephosphorylation of partially autophosphorylated (Ser-63 only) and highly autophosphorylated (Ser-63 and Ser-79) type Iβ PKG by phosphoprotein phosphatase-1 in the presence and absence of cyclic nucleotide was compared. It can be seen that both cGMP and cAMP sharply increased the rate of dephosphorylation of partially autophosphorylated (Ser-63 only) enzyme (Fig. 5*A*), but neither cGMP nor cAMP significantly altered dephosphorylation of the highly autophosphorylated enzyme, *i.e.* enzyme that was autophosphorylated at both Ser-63 and Ser-79 (Fig. 5*B*). The dephosphorylation of PKG that was autophosphorylated only at Ser-63 was stimulated 3–5 fold in 0.5–1.5 min. (Fig. 5*A*). Therefore, using highly autophosphorylated PKG (Fig. 5*B*), it is unlikely that the stimulatory effect of cGMP on phospho-serine 63 would be masked by the absence of an effect of cGMP on dephosphorylation of phospho-serine 79. When highly autophosphorylated PKG was used as substrate, the relative rates of dephosphorylation of phospho-serine 63 and phospho-serine 79 by phosphoprotein phosphatase-1 were comparable as assessed by thin layer chromatography of the phosphopeptides (not shown). It is of interest that the dephosphorylation rate appears to be greater when using the partially autophosphorylated PKG as substrate, but whether this is due to an inhibitory effect of phospho-serine 79 on the dephosphorylation rate requires further study. The data suggest that activation of the PKG by ligand binding (cGMP or cAMP) or by autophosphorylation (both Ser-63 and Ser-79) produces a similar conformational change. Thus, an effect of either cGMP or cAMP on dephosphorylation would be expected only when using the enzyme that is in the inactive conformation (phosphorylated at Ser-63 only). These results using a predominantly enzymatic approach are consistent with those using the chromatographic and electrophoretic approaches.

Concluding Remarks—It should be emphasized that the techniques developed here to resolve cGMP-bound and -free PKG, or phosphorylated and unphosphorylated PKG, could be used for crude systems as well as for purified PKG. The approach of measuring changes in the protein phosphatase sensitivity of the PKG that has been activated by different processes is also novel. Therefore, the techniques offer new approaches for studies of interconversion of these forms of PKG in intact tissues treated with various modulators. These techniques may also be useful in studying other proteins (33), including homologous protein kinases that are activated by ligands or autophosphorylation. In the present studies, autophosphorylation of PKG caused an apparent conformational change that is similar to the elongation of PKG that is produced by cGMP binding (2, 3); this has been demonstrated using each of three separation procedures. The finding that cGMP does not enhance the protein phosphoprotein phosphatase-1 sensitivity

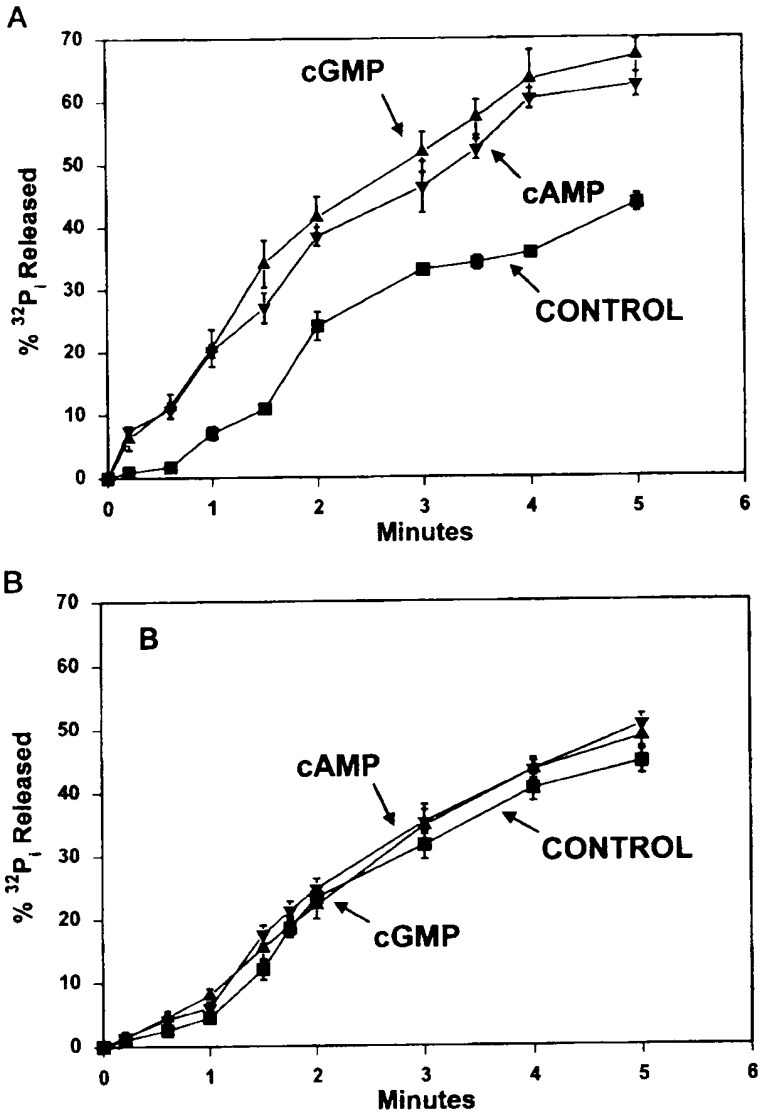

FIG. 5. **Effect of cGMP and cAMP on dephosphorylation of partially and highly autophosphorylated type Iβ PKG with phosphoprotein phosphatase-1.** *A*, 1 μM partially autophosphorylated type Iβ PKG was dephosphorylated with 0.025 units/ml phosphoprotein phosphatase-1 in the absence and presence of 15 μM cGMP or 50 μM cAMP as described in "Experimental Procedures." *B*, 1 μM highly autophosphorylated type Iβ PKG was dephosphorylated with 0.025 unit/ml phosphoprotein phosphatase-1 in the absence and presence of 15 μM cGMP or 50 μM cAMP. Each value represents the mean ± S.D.

of the highly autophosphorylated PKG is consistent with this conclusion.

Either autophosphorylation or cyclic nucleotide binding, or a combination of these processes, can activate catalysis in cyclic nucleotide-dependent protein kinases (4–18), and the two processes appear to produce a similar conformational change in the PKG. This induced structural change in the PKG is associated with conversion of the enzyme from a more compact inactive conformation to a more elongated active conformation and may represent the classical interconversion of enzymes between two states, *i.e.* an inactive T state that has low affinity for substrates and the active R state that has high affinity for substrates (34). Phosphorylase provides one example of the conversion of an enzyme from the T state to the R state, and this is effected by either a phosphorylation event or by ligand binding, *i.e.* 5′-AMP (35). The active conformation that is produced by either process is essentially the same. It is suggested that either cGMP binding or autophosphorylation produces a similar perturbation to cause activation in each monomer of dimeric PKG. This perturbation within the monomers results in an elongation of the dimeric structure. The results of the present study are consistent with such an interconversion in PKG. Since many protein kinases are activated by both ligands and autophosphorylation, these findings could be relevant to the activation mechanism for some of these enzymes as well.

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